

A Proton Magnetic Resonance Study of the Association of Lysozyme with Monosaccharide Inhibitors*

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SUMMARY

It has been shown that the acetamido methyl protons of *N*-acetyl-D-glucosamine undergo a chemical shift to higher fields in their proton magnetic resonance spectrum when the inhibitor is bound to lysozyme. The observed chemical shift in the presence of the enzyme is different for the α - and β -anomeric forms of 2-acetamido-2-deoxy-D-glucopyranose indicating either a difference in the affinity of the anomeric forms for lysozyme or different magnetic environments for the methyl protons in their enzyme-bound state. That the α - and β -anomeric forms of GlcAc bind to lysozyme in a competitive fashion was indicated by observing the proton magnetic resonance spectra in the presence of 2-acetamido-D₃-2-deoxy- α -D-glucopyranose. The methyl glycosides, methyl- α -GlcAc and methyl- β -GlcAc, were also shown to bind competitively with both anomers of GlcAc. Quantitative analysis of the chemical shift data observed for the association of GlcAc with lysozyme was complicated by the mutarotation of GlcAc between its α - and β -anomeric forms. However, in the case of the methyl glucosides, where the conformation of each anomer is frozen, it was possible to analyze the chemical shift data in a straightforward manner, and the dissociation constant as well as the chemical shift of the acetamido methyl protons of the enzyme-inhibitor complex was determined for both anomers. The results indicate that the two anomers of methyl-GlcAc bind to lysozyme with slightly different affinities but that the acetamido methyl groups of both anomers experience identical magnetic environments in the enzyme-inhibitor complex.

Many physical tools have been used to investigate enzyme-substrate and enzyme-inhibitor interactions in solution. In

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general, most of these studies do not yield detailed information regarding the nature of such interactions. Nuclear magnetic resonance spectroscopy, however, by virtue of its extreme sensitivity to minute structural and conformational changes within molecules and to environmental effects, promises to be a potentially important spectroscopic method for the study of such processes.

Most of the results reported so far (1, 2) have been concerned with linewidth changes of the nuclear resonances of the small molecule upon association with the macromolecule. This method has been used to study the binding of penicillin G to bovine serum albumin (2) and the association of ethanol and DPN to an alcohol dehydrogenase (3, 4). Selective broadening of some of the proton resonances of the small molecules has been observed and has been interpreted as indicating intimate contact between such protons (or neighboring groups) and the macromolecule.

In general, one also expects chemical shift changes for some nuclei of the inhibitor or substrate molecule when it is bound to the enzyme. If the small molecule exchanges rapidly between the free and complexed environments, the observed spectral positions of the affected resonances will be shifted from their corresponding positions for the unassociated molecule by amounts which depend upon the chemical shifts of these nuclei in the complex as well as on the fraction of the inhibitor or substrate molecules in the complexed state. One might argue that the chemical shift changes are more *stereospecific* than the linewidth changes in these studies, and hence provide a more sensitive probe of the active site and the nature of the enzyme-substrate or enzyme-inhibitor interactions. However, the chemical shift changes are in general considerably smaller than the accompanying broadening of the resonances and are therefore more difficult to measure.

Since the work described in this communication was started,¹ a preliminary account of a study of the association of 2-acetamido-2-deoxy-D-glucopyranide and related compounds with

¹ A preliminary account of the work described here was presented at the Gordon Research Conference on Proteins, New Hampton School, New Hampshire, June 27, 1966. Preliminary reports of similar proton magnetic resonance studies on lysozyme (5) and chymotrypsin (6) association with inhibitors have appeared.

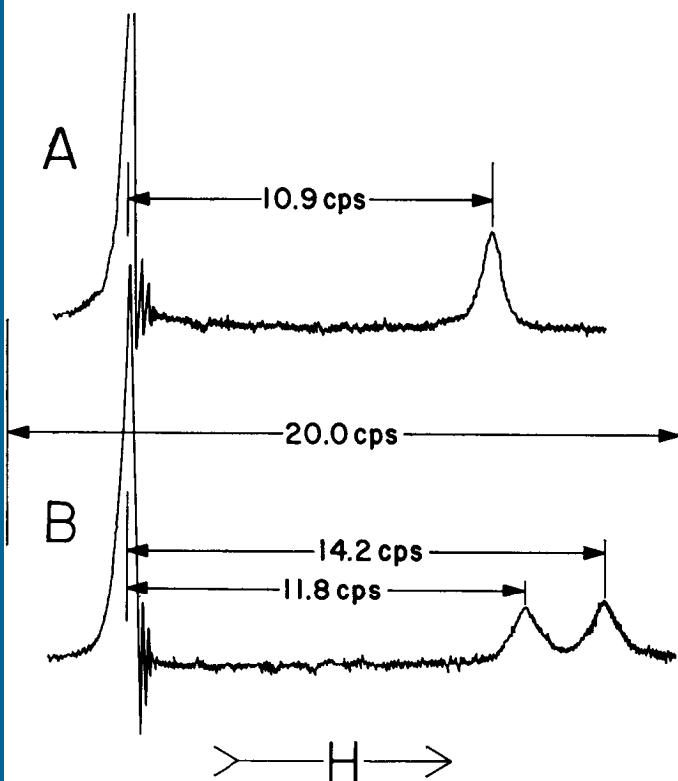


Fig. 1. A, proton magnetic resonance spectrum at 60 MHz of the acetamido methyl protons of GlcAc (5×10^{-2} M) in 0.1 M citrate, pH 5.5. Acetone (0.5%) is used as an internal standard and its resonance appears to lowest field in spectrum. A sweep width of 50 cps was used. B, proton magnetic resonance spectrum at 60 MHz of a similar solution of GlcAc as shown in A with added lysozyme (3×10^{-3} M).

lysozyme as detected by PMR² methods has appeared (5). In addition to line-broadening effects caused by binding, it was observed that the resonance of the acetamido methyl groups of various GlcAc derivatives were shifted to higher field in the presence of lysozyme. This effect was observed for α -GlcAc, β -GlcAc, methyl- α -GlcAc, methyl- β -GlcAc, and *N*-acetyl-D-galactosamine.

In this communication, we present the results of an investigation of the chemical shift changes occurring in the association of lysozyme with some specific inhibitors. The emphasis of this work is on the quantitation of such chemical shifts and the interpretation of the results obtained to infer information regarding the microscopic environment experienced by the inhibitor when bound to the enzyme.

MATERIALS AND METHODS

N-Acetyl-D-glucosamine (m.p. 203–205°) was purchased from Calbiochem. *N*-Acetyl-D₃-glucosamine was obtained by acetylation of D-glucosamine-HCl (Calbiochem) with acetic anhydride-*d*₆ (Volk Radiochemical Company (Chicago)) according to published procedures (7). Recrystallization of the resulting *N*-acetyl(D₃)- α -D-glucopyranose was effected from aqueous-alcohol mixtures (m.p. 203–205°). Methyl-2-acetamido-2-deoxy- α -D-glucopyranoside (m.p. 188°) was synthesized as

² The abbreviations used are: PMR, proton magnetic resonance; methyl- α -GlcAc, methyl-2-acetamido-2-deoxy- α -D-glucopyranoside; methyl- β -GlcAc, methyl-2-acetamido-2-deoxy- β -D-glucopyranoside; GlcAc, 2-acetamido-2-deoxy-D-glucopyranide.

previously reported (8) and purified by chromatography on a charcoal-Celite column. Methyl-2-acetamido-2-deoxy- β -D-glucopyranoside (m.p. 204–205°) was synthesized (9) from D-glucosamine. Lysozyme (Lot 668-8590) was obtained from Sigma.

The 60-MHz PMR spectra were recorded on a Varian A-60A spectrometer at a probe temperature of 40°. A Varian HA-100 spectrometer, operating in frequency sweep mode, was used for the 100-MHz PMR spectra, which were measured at 30°. The water resonance was used as a lock signal for the studies in H₂O while a capillary of TMS was used in the studies conducted in D₂O.

The chemical shifts for the 100-MHz spectra were obtained by counting the frequency difference between the manual oscillator and the sweep oscillator on the HA-100 spectrometer with a Hewlett Packard model 5212A counter. All chemical shifts were measured relative to an internal standard of acetone (0.5%). In each case, except those for mutarotational studies, the chemical shifts were measured at least three times with a standard deviation of 0.04 cps or less. Data were analyzed by methods of least squares.

Enzyme concentrations of samples used for the PMR measurements were determined from ultraviolet absorbance at 280 m μ of a 25- μ l aliquot, after dilution to 5 ml with water, with the use of the known extinction coefficient (9) for lysozyme.

RESULTS

The PMR spectrum at 60 MHz of *N*-acetyl-D-glucosamine exhibits a resonance at τ 8.09, which can be unambiguously assigned to the methyl group protons of the acetamido side chain (10). Because it is the most intense resonance in the spectrum and is unsplit, it is best suited for our present study. Fig. 1A shows this resonance at 60 MHz with an acetone internal standard included in the sample. The addition of lysozyme (3×10^{-3} M final concentration) to this same sample of GlcAc (5×10^{-2} M) resulted in the spectrum shown in Fig. 1B. From the known binding constant (11), it was determined that approximately 5% of the total GlcAc present was bound to the enzyme under these conditions. Two resonances instead of one were observed for these acetamido methyl protons in the presence of the enzyme. Both of these resonances were broadened, and both appeared at higher fields from the position of the original GlcAc methyl resonance (0.9 Hz and 3.3 Hz, respectively).

As proof that the effects observed in the PMR spectra of GlcAc in the presence of lysozyme were due to association between the inhibitor and the enzyme rather than to bulk susceptibility effects in the relatively concentrated protein solution, the PMR spectra of GlcAc in similarly concentrated solutions of ribonuclease A were also recorded. Such a spectrum is displayed in Fig. 2. In addition to the absence of the additional resonance observed in the presence of lysozyme, it was shown that the addition of ribonuclease to a GlcAc solution did not result in any chemical shift of the acetamido methyl resonance from its position in free GlcAc. Neither was the methyl resonance noticeably broadened. It was further substantiated that the concentration of GlcAc itself did not affect the chemical shift of its acetamido methyl protons. No concentration shifts were observed over the concentration range 10^{-2} M to 10^{-1} M.

To ascertain that the two observed methyl resonances of GlcAc in the presence of lysozyme correspond to the acetamido methyl groups of the α - and β -anomeric forms of the inhibitor a freshly dissolved sample of α -GlcAc was used. Fig. 3 summarizes the

PMR spectra of the GlcAc methyl protons in the presence of the enzyme obtained at various intervals during the mutarotation of GlcAc from the α -anomeric form to the equilibrium mixture of α and β anomers. Lysozyme was also added to a fresh sample of β -GlcAc prepared by acetylation of β -D-glucosamine (12). The spectrum for this solution showed at early times only the methyl resonance which we have attributed to the β anomer in Fig. 3, with subsequent development of the resonance due to the α anomer.

These observations are in agreement with those previously observed (5) for GlcAc in the presence of lysozyme, and they are also in accord with the results obtained from x-ray analysis studies (13) of lysozyme-GlcAc complexes in the crystalline state which have shown that both anomers of the sugar bind to the enzyme. Earlier studies in this laboratory (11) with ultraviolet spectroscopic methods allowed estimation of the dissociation constant for the lysozyme-GlcAc complex ($K_s = 4$ to 6×10^{-2} M). This value obviously represents the binding of both forms of GlcAc and is a complex constant.

It is evident from our present observations that the chemical exchange of both anomeric forms of the inhibitor GlcAc between the free and enzyme bound species is rapid. It is also evident that both anomers of GlcAc, when bound to lysozyme have their acetamido methyl groups in environments which lead to increased magnetic shielding of these protons, since the resonances observed for both anomers are shifted upfield from their positions in the absence of the enzyme. The fact that we observe separate resonances for the α and β anomers in the presence of lysozyme indicates that the two anomeric forms bind to the enzyme either with different affinities, or in a manner such that their acetamido side chains do not occupy the same position on the enzyme or both. These conclusions are possible, whether or not the two anomers bind to different sites or compete for the same sites on the enzyme surface. In addition, the rate of interconversion of GlcAc between its α and β anomers must be slow.

To decide whether the α - and β -anomeric forms of GlcAc

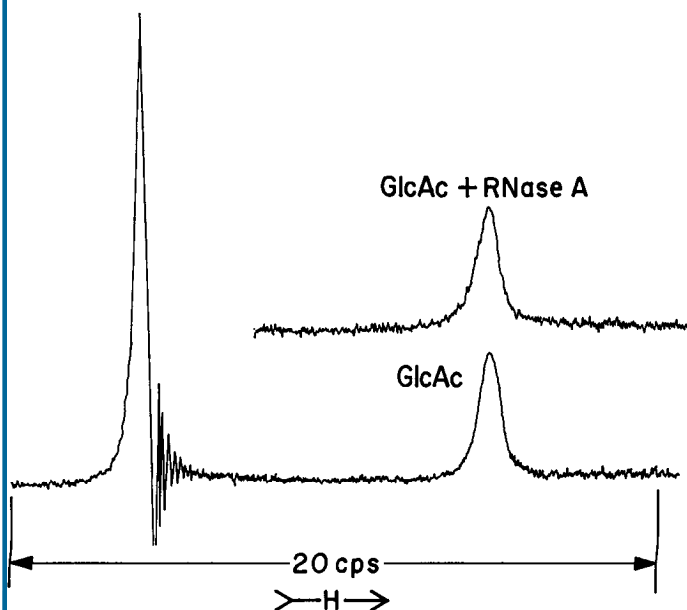


Fig. 2. The acetamido methyl PMR spectrum at 60 MHz of GlcAc (5×10^{-2} M) in the absence and in the presence of ribonuclease A (3×10^{-3} M), in 0.1 M citrate, pH 5.5. Acetone (0.5%) was used as an internal reference, and appears to lower field.

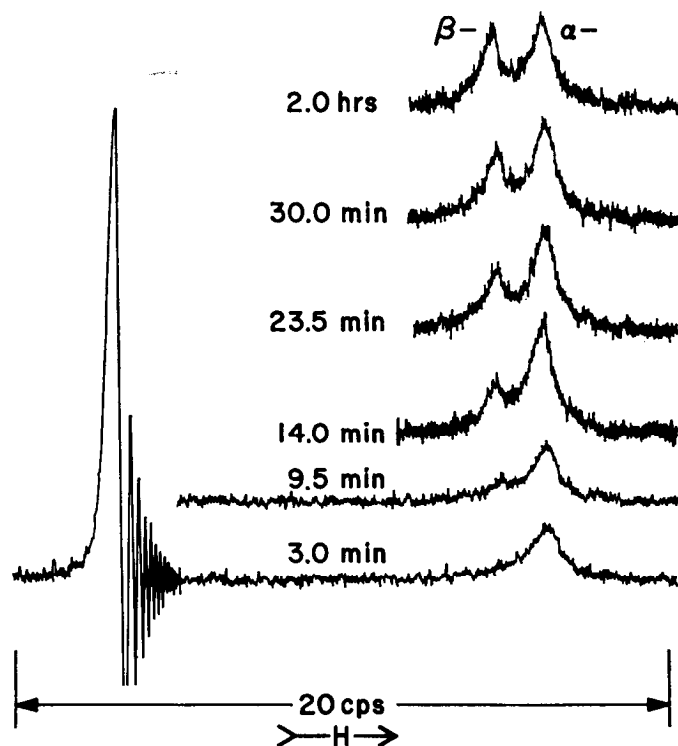


Fig. 3. Time study of the PMR spectrum of α -GlcAc (5×10^{-2} M) after addition to lysozyme (3×10^{-3} M) in 0.1 M citrate in D_2O , pH 5.9. A solution of acetone (0.5%) was used as an internal standard, and its resonance appears to lower field of the GlcAc resonances.

compete for the same sites on the enzyme surface, a sample of crystalline *N*-acetyl(d_3)- α -D-glucopyranose (m.p. 203–205°) was added to an equilibrated mixture of GlcAc and lysozyme and the PMR spectrum was recorded at 60 MHz within 2 min after mixing. As seen in Fig. 4, the added α -(d_3) anomer (although not observable) decreased the observed chemical shifts (and therefore the percentage bound) of both α - and β -GlcAc. Table I shows the quantitative effects observed. As can be seen from Table I, both anomers were approximately equally affected by the added α -(d_3) anomer. The conclusion from this experiment is that α -GlcAc and β -GlcAc do compete for the same sites on the enzyme.

A quantitative analysis of the chemical shift data obtained for the GlcAc-lysozyme system is complicated by the mutarotation equilibrium between the α and β anomers of the inhibitor, since the enzyme would shift this equilibrium if the two anomeric forms bind to the enzyme with different affinities. For facilitation of quantitative treatment of the experimental data, identical binding studies were therefore repeated with the methyl glycosides of GlcAc, where the conformation of each anomer is frozen. Table II summarizes the results obtained at 100 MHz for the association of lysozyme with methyl-*N*-acetyl- β -D-glucopyranoside and separately with methyl-*N*-acetyl- α -D-glucopyranoside.

That methyl- α -GlcAc and methyl- β -GlcAc compete for the same sites on the enzyme surface was shown by adding *N*-acetyl(d_3)- α -D-glucopyranose to a solution of lysozyme containing either methyl- α -GlcAc or methyl- β -GlcAc, and showing that the added deuterated compound reduced the amount of bound methyl- α -GlcAc or methyl- β -GlcAc. Table III gives the quantitative data obtained. Methyl- α -GlcAc and methyl-

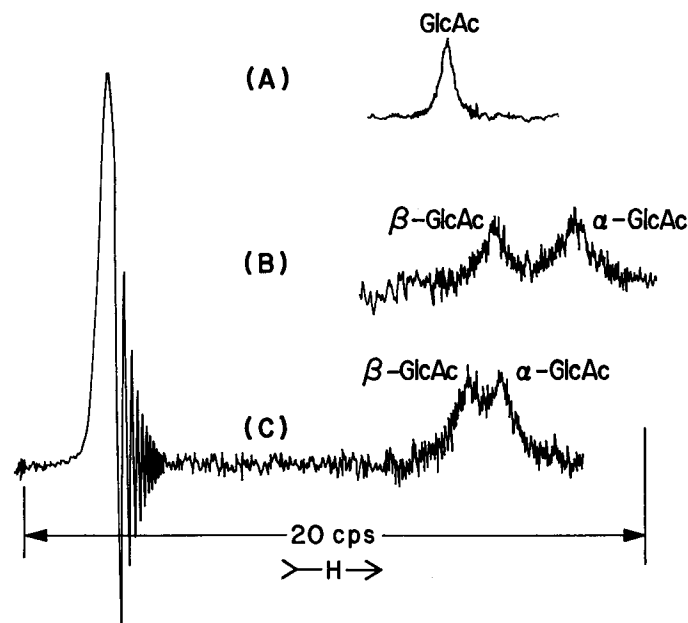


FIG. 4. Competitive binding to lysozyme of α -GlcAc and β -GlcAc. The resonance caused by acetone protons is shown at left. Other resonances shown are (A) the acetamido methyl proton resonance of free GlcAc; (B) GlcAc (at mutarotation equilibrium, 1.6×10^{-2} M) in the presence of lysozyme; (C) same as in B but with added α -GlcAc(d_3) (6.7×10^{-2} M). In this case, the spectrum was recorded immediately after addition of the α -deuterio anomer.

TABLE I

Chemical shift data for acetamido methyl protons of α -GlcAc and β -GlcAc association with lysozyme in absence and presence of α -GlcAc(d_3)

Measurements were made at 60 MHz in 0.1 M citrate buffer, pH 5.5, at 40°.

Inhibitor	Concentration		Chemical shift relative to acetone	δ
	Inhibitor	Lysozyme		
	<i>M</i>	<i>mg/ml</i>	<i>Hz</i>	
α -, β -GlcAc	0.05	0	10.92	0
α -GlcAc	0.008	50	14.76	3.84
β -GlcAc	0.008		12.24	1.32
α -GlcAc	0.008	50	12.54	1.62
β -GlcAc	0.008		11.50	0.58
α -GlcAc(<i>d</i> ₃)	0.067			

β -GlcAc compete for the same sites on the enzyme occupied by α -GlcAc.

The results summarized in Table II can be readily interpreted in terms of the following simple equilibrium between the enzyme (E) and the inhibitor (S),



Under conditions of rapid chemical exchange of the inhibitor between its free and enzyme-bound forms, it is readily shown that

$$K_s \frac{\delta}{\Delta} = \left(E_0 - S_0 \frac{\delta}{\Delta} \right) \left(1 - \frac{\delta}{\Delta} \right) \quad (2)$$

where δ is the observed chemical shift of the average methyl resonance referred to that of the unbound inhibitor, Δ is the chemical shift difference for the methyl resonance between the bound and unbound species, K_s is the enzyme-inhibitor dissociation constant, and E_0 and S_0 denote the total concentrations of the enzyme and inhibitor, respectively. In the limit where $\delta/\Delta \ll 1$, a condition which is fairly well satisfied in our experiments, Expression 2 simplifies and may be rearranged to give

$$S_0 = E_0 \frac{\Delta}{\delta} - K_s - E_0 \quad (3)$$

Thus, if the enzyme-inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of enzyme, a plot of S_0 with respect to the observed $1/\delta$ limits should yield a straight line with slope $E_0\Delta$ and intercept equal to $-(K_s + E_0)$, provided the above conditions are met.

The chemical shift data obtained for methyl- α - and methyl- β -GlcAc are plotted in this manner in Fig. 5. In these experi-

TABLE II

Chemical shift data for acetamido methyl protons of methyl- α -GlcAc and methyl- β -GlcAc association with lysozyme (2.0×10^{-3} M)

Measurements were made at 100 MHz, in 0.1 M citrate buffer, pH 5.5, at 31°.

$S_0 \times 10^2$	δ	$1/\delta$
	Hz	
Methyl- α -GlcAc		
7.15	1.30	0.769
4.29	1.69	0.592
3.57	1.82	0.549
2.86	1.95	0.513
1.43	2.39	0.418
Methyl- β -GlcAc		
7.21	1.45	0.690
5.77	1.71	0.585
4.33	1.95	0.513
3.60	2.20	0.454
2.88	2.43	0.412
1.44	3.13	0.320

TABLE III

Chemical shift data for acetamido methyl protons obtained from association of methyl- α -GlcAc and methyl- β -GlcAc with lysozyme in absence and presence of α -GlcAc(d_3)

Measurements were made in 0.1 M citrate buffer, pH 5.5, at 40°, in a Varian A-60A spectrometer.

Inhibitor	Concentration		Chemical shift relative to acetone	δ
	Inhibitor	Lysozyme		
	<i>M</i>	<i>mg/ml</i>	<i>Hz</i>	
Methyl- α -GlcAc	0.05	0	11.52	0
Methyl- α -GlcAc	0.016	50	13.12	1.60
Methyl- α -GlcAc	0.016	50	12.76	1.24
plus				
α -GlcAc(d_3)	0.019			
Methyl- β -GlcAc	0.05	0	11.54	0
Methyl- β -GlcAc	0.016	50	13.84	2.30
Methyl- β -GlcAc	0.016	50	13.18	1.64
plus				
α -GlcAc(d_3)	0.023			

ments, E_0 was held fixed at 3.0×10^{-3} M. It is seen that the variation of S_0 with $1/\delta$ is indeed linear for both anomeric methyl glycosides over the range of conditions of our enzyme-inhibitor binding experiments. From an analysis of these plots, we extracted the following dissociation constants and chemical shifts for the two enzyme-glycoside complexes:

$$K_{\alpha} = 5.2 \pm 0.4 \times 10^{-2} \text{ M}, \quad \Delta_{\alpha} = 0.55 \pm 0.02 \text{ ppm}$$

$$K_{\beta} = 3.3 \pm 0.5 \times 10^{-2} \text{ M}, \quad \Delta_{\beta} = 0.54 \pm 0.04 \text{ ppm}$$

Thus, it appears that the two anomeric glycosides bind to lysozyme with slightly different affinities. However, the results indicate that the acetamido methyl groups of both glycosides occupy identical magnetic environments when bound to the enzyme.

The results of the above analysis indicate that only one inhibitor molecule is bound per lysozyme molecule. Thus, if there is more than one comparably strong binding site on the enzyme surface, it is apparent that the formation of one enzyme-inhibitor complex inhibits binding at the remaining sites. As usual, it is not possible to establish the formation of one 1:1 enzyme-inhibitor complex rather than several such complexes, irrespective of whether binding at one or several sites is responsible for the observed spectral changes. The reason for this is that the concentration of the various enzyme-inhibitor complexes are related to one another merely by ratios of their formation constants. In our case, where the various 1:1 enzyme-inhibitor complexes would be in rapid equilibrium with one another, the expression for the observed chemical shift can be reduced to the same form as Equation 2, with K_i and Δ replaced by their appropriate weighted averages, namely

$$K_s \rightarrow 1 / \left(\sum_{i=1}^n 1/K_{s_i} \right)$$

$$\Delta \rightarrow \left(\sum_{i=1}^n \Delta_i / K_{s_i} \right) / \left(\sum_{i=1}^n 1/K_{s_i} \right) \quad (4)$$

Here, n denotes the number of binding sites, and the subscript i is used to denote the parameters for the i th enzyme-inhibitor complex.

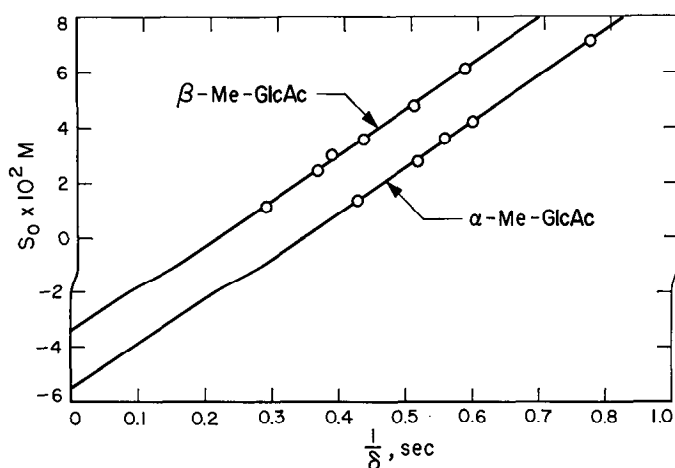


Fig. 5. Plots of chemical shift (σ) data for the association of methyl- α -GlcAc (α -Me-GlcAc) and separately methyl- β -GlcAc (β -Me-GlcAc) with lysozyme. The ordinate showing $S_0 \times 10^2$ refers to the concentration of each inhibitor used (1×10^{-2} to 7×10^{-2} M) in the presence of a constant concentration of the enzyme (3×10^{-3} M).

DISCUSSION

The most reasonable explanation of the observed shifts for the acetamido methyl protons on the binding of acetamido sugars to lysozyme is that these protons become more magnetically shielded in the enzyme-bound state because of their environment on the enzyme. The most likely causes for this are proximity of the methyl group to (a) an aromatic side chain of the enzyme (14), or (b) the electric field of an ionizable group nearby (15). There is ample evidence (16) that the binding of *N*-acetyl-D-glucosamine and related compounds to lysozyme results in a red shift in the spectrum of the enzyme, and this observation has been attributed to a tryptophanyl side chain becoming less accessible to solvent. In addition, x-ray studies (5, 17) of the binding of small sugar molecules such as GlcAc to crystalline lysozyme suggest that in the enzyme-inhibitor complex, the acetamido methyl group is close to the aromatic ring of tryptophane residue 108. There is, however, also evidence that ionizable groups are present at the binding site of lysozyme (18, 19).

The results of this work indicate that the acetamido methyl protons of methyl- α -GlcAc and methyl- β -GlcAc experience identical magnetic environments in the enzyme-inhibitor complex. This conclusion is not true in the case of GlcAc where both binding constants and chemical shifts for the anomeric forms have been shown to be different from each other (20). On the other hand, in the methyl glycosides, the glycosidic methyl groups of methyl- α -GlcAc and methyl- β -GlcAc were found to experience different magnetic environments when bound to the enzyme. In the presence of lysozyme, this methyl resonance was shifted to lower fields in methyl- β -GlcAc, whereas in the case of methyl- α -GlcAc, the glycosidic methyl resonance was not noticeably affected. Of the other protons in the GlcAc molecule, only those of the 6-hydroxymethyl group were investigated in the presence of lysozyme. No observable chemical shift of these protons was induced by the enzyme, whereas broadening of the resonances was observed, as expected.

We have also been able to show³ that other acetamido sugars bind to the same site as do the various GlcAc derivatives. Such compounds as *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, acetamidocyclohexanol, and acetamidocyclohexane all interact with the same site on the enzyme and the methyl group of the acetamido side chain undergoes a chemical shift in each case. This is consistent with the idea that the acetamido side chain is necessary for binding to the enzyme to occur, probably through the formation of hydrogen bonds. All the compounds studied probably form the same hydrogen bonds between the enzyme and the acetamido group of the inhibitor, but interactions at other "points of contact" between the enzyme and inhibitor molecules may differ depending on the nature of the substituents and their conformation on each pyranose ring.

It would appear from our results that there is at least one strong binding site on the enzyme for simple acetamido pyranosides, such as GlcAc. However, it appears that if there is more than one strong binding site on the enzyme surface, the formation of one enzyme-inhibitor complex inhibits binding at the remaining sites, so that only 1:1 complexes result. This conclusion that only 1 inhibitor molecule is bound strongly per lysozyme molecule agrees with previous spectrophotometric

³ S. M. Parsons and M. Raftery, unpublished observations.

studies in this laboratory (12) and elsewhere (19, 21). However, these spectrophotometric studies also suggest that there are three contiguous binding sites for pyranose rings on the enzyme surface, since the binding strength of oligosaccharides to lysozyme was found to increase up to the trisaccharide of GlcAc (chitotriose).

It is felt that these studies show that the use of PMR spectroscopy offers advantages over methods previously used for the study of enzyme-inhibitor or enzyme-substrate interactions, inasmuch as information regarding the environment experienced by the small molecule on association with the macromolecule can often be obtained.

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